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Description

The invention relates to a method for the fermentative production of sulfur-containing fine chemicals, in particular L-methionine, by using bacteria which express a nucleotide sequence
5 coding for an O-acetylhomoserine sulfhydrylase (metY) gene.

Prior art

Sulfur-containing fine chemicals such as, for example, methionine, homocysteine,
10 S-adenosylmethionine, glutathione, cysteine, biotin, thiamine, lipoic acid are produced in cells via natural metabolic processes and are used in many branches of industry, including the food, animal feed, cosmetics and pharmaceutical industries. These substances which are collectively referred to "sulfur-containing fine chemicals" include organic acids, both proteinogenic and nonproteinogenic amino acids, vitamins and cofactors. They are most expediently produced on
15 a large scale by means of cultivating bacteria which have been developed in order to produce and secrete large amounts of the substance desired in each case. Organisms which are particularly suitable for this purpose are coryneform bacteria, Gram-positive nonpathogenic bacteria.

20 It is known that amino acids are produced by fermentation of strains of coryneform bacteria, in particular Corynebacterium glutamicum. Due to the great importance, the production processes are constantly improved. Process improvements can relate to measures regarding technical aspects of the fermentation, such as, for example, stirring and oxygen supply, or to the nutrient media composition such as, for example, sugar concentration during fermentation or to the work-
25 up to give the product, for example by ion exchange chromatography, or to the intrinsic performance properties of the microorganism itself.

A number of mutant strains which produce an assortment of desirable compounds from the group of sulfur-containing fine chemicals have been developed via strain selection. The
30 performance properties of said microorganisms are improved with respect to the production of a particular molecule by applying methods of mutagenesis, selection and mutant selection. However, this is a time-consuming and difficult process. In this way strains are obtained, for example, which are resistant to antimetabolites such as, for example, the methionine analogs α -methylmethionine, ethionine, norleucine, n-acetylnorleucine, S-trifluoromethylhomocysteine,

2-amino-5-heprenoitic acid, selenomethionine, methioninesulfoximine, methoxine, 1-amino-cyclopentanecarboxylic acid or which are auxotrophic for metabolites important for regulation and which produce sulfur-containing fine chemicals such as, for example, L-methionine.

- 5 Methods of recombinant DNA technology have also been used for some years to improve Corynebacterium strains producing L-amino acids by amplifying individual amino-acid biosynthesis genes and investigating the effect on amino acid production.

10 WO-A-02/18613 describes the nucleic acid sequence and the amino acid sequence for metY from C. glutamicum and its use for the production of L-lysine.

Brief description of the invention

15 It is an object of the present invention to provide a novel method for the improved fermentative production of sulfur-containing fine chemicals, in particular L-methionine.

We have found that this object is achieved by providing a method for the fermentative production of a sulfur-containing fine chemical, comprising the expression of a heterologous nucleotide sequence coding for a protein with metY activity in a coryneform bacterium.

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The invention firstly relates to a method for the fermentative production of at least one sulfur-containing fine chemical, which comprises the following steps:

- 25 a) fermentation of a coryneform bacteria culture producing the desired sulfur-containing fine chemical, the coryneform bacteria expressing at least one heterologous nucleotide sequence which codes for a protein with O-acetylhomoserine sulfhydrylase (metY) activity;
- b) concentration of the sulfur-containing fine chemical in the medium or in the bacterial cells, and
- 30 c) isolation of the sulfur-containing fine chemical, which preferably comprises L-methionine.

The above heterologous metY-encoding nucleotide sequence is preferably less than 100%, such as, for example, more than 70%, such as 75, 80, 85, 90 or 95%, or less than 70%, such as, for example, up to 60, 50, 40, 30, 20 or 10% homologous to the metY-encoding sequence from Corynebacterium glutamicum ATCC 13032. The metY-encoding sequence is derived preferably

from any of the following organisms of list I:

List I

<i>Corynebacterium diptheriae</i>	ATCC 14779
<i>Mycobacterium tuberculosis</i> CDC1551	ATCC 25584
<i>Clostridium acetobutylicum</i>	ATCC 824
<i>Bacillus halodurans</i>	ATCC21591
<i>Bacillus stearothermophilus</i>	ATCC 12980
<i>Chlorobium tepidum</i>	ATCC 49652
<i>Synechococcus</i> sp.	ATCC27104
<i>Emericella nidulans</i>	ATCC 36104
<i>Bacteroides fragilis</i>	ATCC 25285
<i>Lactococcus lactis</i>	ATCC 7962
<i>Bordetella bronchiseptica</i>	ATCC 19395
<i>Pseudomonas aeruginosa</i>	ATCC 17933
<i>Nitrosomonas europaea</i>	ATCC 19718
<i>Sinorhizobium meliloti</i>	ATCC 4399
<i>Thermotoga maritima</i>	ATCC 43589
<i>Streptococcus mutans</i>	ATCC 25175
<i>Burkholderia cepacia</i>	ATCC 25416
<i>Deinococcus radiodurans</i>	ATCC 13939
<i>Rhodobacter capsulatus</i>	ATCC 11166
<i>Pasteurella multocida</i>	ATCC 6530
<i>Clostridium difficile</i>	ATCC 9689
<i>Campylobacter jejuni</i>	ATCC 33560
<i>Streptococcus pneumoniae</i>	ATCC 6308
<i>Saccharomyces cerevisiae</i>	ATCC 2704
<i>Kluyveromyces lactis</i>	ATCC 8585
<i>Candida albicans</i>	ATCC 10231
<i>Schizosaccharomyces pombe</i>	ATCC 24969

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ATCC: American Type Culture Collection, Rockville, MD, USA

The metY-encoding sequence used according to the invention preferably comprises a coding sequence according to SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51 and 53 or a nucleotide sequence homologous thereto which codes for a protein with metY activity.

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Moreover, the metY-encoding sequence used according to the invention preferably codes for a protein with metY activity, said protein comprising an amino acid sequence according to SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52 and 54 or an amino acid sequence homologous thereto which represents a protein with metY activity.

The coding metY sequence is preferably a DNA or an RNA which can be replicated in coryneform bacteria or is stably integrated into the chromosome.

10 According to a preferred embodiment, the method of the invention is carried out by

a) using a bacterial strain transformed with a plasmid vector which carries at least one copy of the coding metY sequence under the control of regulatory sequences or

b) using a strain in which the coding metY sequence has been integrated into the bacterial chromosome.

Furthermore, preference is given to overexpressing the coding metY sequence for the fermentation.

20 It may also be desirable to ferment bacteria in which additionally at least one further gene of the biosynthetic pathway of the desired sulfur-containing fine chemical has been amplified; and/or in which at least one metabolic pathway, which reduces production of the desired sulfur-containing fine chemical has, at least partially, been switched off.

25 It may also be desirable to ferment bacteria in which additionally the activity of at least one further gene of the biosynthetic pathway of the desired sulfur-containing fine chemical is not undesirably influenced by metabolic metabolites.

Therefore, according to another embodiment of the method of the invention, coryneform bacteria are fermented in which, at the same time, at least one of the genes selected from among

- a) the gene lysC, which encodes an aspartate kinase,
- b) the gene asd, which encodes an aspartate-semialdehyde dehydrogenase,
- c) the glyceraldehyde-3-phosphate dehydrogenase-encoding gene gap,

- d) the 3-phosphoglycerate kinase-encoding gene pgk,
- e) the pyruvate carboxylase-encoding gene pyc,
- f) the triose phosphate isomerase-encoding gene tpi,
- g) the homoserine O-acetyltransferase-encoding gene metA,
- 5 h) the cystathionine gamma-synthase-encoding gene metB,
- i) the cystathionine gamma-lyase-encoding gene metC,
- j) the serine hydroxymethyltransferase-encoding gene glyA,
- k) the methionine synthase-encoding gene methH,
- l) the methylene tetrahydrofolate reductase-encoding gene metF,
- 10 m) the phosphoserine aminotransferase-encoding gene serC,
- n) the phosphoserine phosphatase-encoding gene serB,
- o) the serine acetyl transferase-encoding gene cysE,
- p) the homoserine dehydrogenase-encoding gene hom

is overexpressed.

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According to another embodiment of the method of the invention, coryneform bacteria are fermented in which, at the same time, at least one of the genes selected from among genes of the abovementioned group a) to p) is mutated in such a way that the activity of the corresponding proteins is influenced by metabolic metabolites to a smaller extent, if at all, compared to
20 nonmutated proteins and that in particular the inventive production of the fine chemical is not adversely affected.

According to another embodiment of the method of the invention, coryneform bacteria are fermented in which, at the same time, at least one of the genes selected from among

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- q) the homoserine kinase-encoding gene thrB,
- r) the threonine dehydratase-encoding gene ilvA,
- s) the threonine synthase-encoding gene thrC,
- t) the meso-diaminopimelate D-dehydrogenase-encoding gene ddh,
- u) the phosphoenolpyruvate carboxykinase-encoding gene pck,
- 30 v) the glucose-6-phosphate 6-isomerase-encoding gene pgi,
- w) the pyruvate oxidase-encoding gene poxB,
- x) the dihydrodipicolinate synthase-encoding gene dapA,
- y) the dihydrodipicolinate reductase-encoding gene dapB; or
- z) the diaminopicolinate decarboxylase-encoding gene lysA

is attenuated, in particular by reducing the rate of expression of the corresponding gene.

According to another embodiment of the method of the invention, coryneform bacteria are fermented in which, at the same time, at least one of the genes of the above groups q) to z) is mutated in such a way that the enzymic activity of the corresponding protein is partially or completely reduced.

Preference is given to using, in the method of the invention, microorganisms of the species *Corynebacterium glutamicum*.

The invention further relates to a method for producing an L-methionine-containing animal feed additive from fermentation broths, which comprises the following steps:

- a) culturing and fermentation of an L-methionine-producing microorganism in a fermentation medium;
- b) removal of water from the L-methionine-containing fermentation broth;
- c) removal of from 0 to 100% by weight of the biomass formed during fermentation; and
- d) drying of the fermentation broth obtained according to b) and/or c), in order to obtain the animal feed additive in the desired powder or granule form.

The invention likewise relates to the coding metY sequences isolated from the above microorganisms for the first time, to the O-acetylhomoserine sulfhydrylases encoded thereby and to the functional homologs of these polynucleotides and proteins, respectively.

Detailed description of the invention

a) General terms

Proteins with O-acetylhomoserine sulfhydrylase activity, also referred to as metY (EC 4.2.99.10), are described as being proteins which are capable of converting O-acetylhomoserine and sulfide into homocystein, using the cofactor pyridoxal phosphate. The skilled worker distinguishes between the activity of O-acetylhomoserine sulfhydrylase and that of O-succinylhomoserine sulfhydrylase, also referred to as metZ. In the latter enzyme, O-succinylhomoserine and not O-acetylhomoserine acts as the substrate for the reaction. The skilled worker can detect the

enzymatic activity of mety by means of enzyme assays, protocols for which may be: Shimizu H. Yamagata S. Masui R. Inoue Y. Shibata T. Yokoyama S. Kuramitsu S. Iwama T. *Biochimica et Biophysica Acta*. 1549(1):61-72, 2001, Yamagata S. Isaji M. Nakamura K. Fujisaki S. Doi K. Bawden S. D'Andrea R. *Applied Microbiology & Biotechnology*. 42(1):92-9, 1994.

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Within the scope of the present invention, the term "sulfur-containing fine chemical" includes any chemical compound which contains at least one covalently bound sulfur atom and is accessible by a fermentation method of the invention. Nonlimiting examples thereof are methionine, homocysteine, S-adenosylmethionine, in particular methionine and S-adenosylmethionine.

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Within the scope of the present invention, the terms "L-methionine", "methionine", homocysteine and S-adenosylmethionine also include the corresponding salts such as, for example, methionine hydrochloride or methionine sulfate.

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"Polynucleotides" in general refers to polyribonucleotides (RNA) and polydeoxyribonucleotides (DNA) which may be unmodified RNA and DNA respectively, or modified RNA and DNA, respectively.

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According to the invention, "polypeptides" means peptides or proteins which contain two or more amino acids linked via peptide bonds.

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The term "metabolic metabolite" refers to chemical compounds which occur in the metabolism of organisms as intermediates or else final products and which, apart from their property as chemical building blocks, may also have a modulating effect on enzymes and on their catalytic activity. It is known from the literature that such metabolic metabolites may act on the activity of enzymes in both an inhibiting and a stimulating manner (Biochemistry, Stryer, Lubert, 1995 W. H. Freeman & Company, New York, New York.). The possibility of producing in organisms enzymes in which the influence of metabolic metabolites has been modified by measures such as mutation of the genomic DNA by UV radiation, ionizing radiation or mutagenic substances and subsequent selection for particular phenotypes has also been described in the literature (Sahm H., Eggeling L., de Graaf AA., *Biological Chemistry* 381(9-10):899-910, 2000; Eikmanns BJ., Eggeling L., Sahm H., *Antonie van Leeuwenhoek*, 64:145-63, 1993-94). These altered properties may also be achieved by specific measurements. The skilled worker knows that it is possible specifically to modify in enzyme genes particular nucleotides of the DNA coding for the

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protein that the protein resulting from the expressed DNA sequence has certain new properties, in such a way for example that the modulating effect of metabolic metabolites on the unmodified protein has changed.

- 5 The activity of enzymes may be influenced in such a way that the reaction rate is reduced or the affinity for the substrate is modified or the reaction rates are changed.

10 The terms "express" and "amplification" or "overexpression" describe in the context of the invention the production of or increase in intracellular activity of one or more enzymes encoded by the corresponding DNA in a microorganism. For this purpose, for example, it is possible to introduce a gene into an organism, to replace an existing gene by another gene, to increase the copy number of the gene or genes, to use a strong promoter or to use a gene which codes for a corresponding enzyme having a high activity, and these measures can be combined, where appropriate.

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b) metY proteins of the invention

The invention likewise includes "functional equivalents" of the specifically disclosed metY enzymes of organisms in the above list I.

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Within the scope of the present invention, "functional equivalents" or analogs of the specifically disclosed polypeptides are polypeptides different therefrom, which furthermore have the desired biological activity such as, for example, substrate specificity.

25 According to the invention, "functional equivalents" means in particular mutants which have in at least one of the abovementioned sequence positions an amino acid other than the specifically mentioned amino acid, but which have nevertheless one of the abovementioned biological activities. "Functional equivalents" thus also include the mutants obtainable by one or more amino acid additions, substitutions, deletions and/or inversions, it being possible for said
30 modifications to occur at any position in the sequence as long as they result in a mutant having the property profile of the invention. There is functional equivalence in particular also when the reaction patterns of mutant and unmodified polypeptide match qualitatively, i.e. identical substrates are converted with different rates, for example.

"Functional equivalents" naturally also comprise polypeptides which are obtainable from other organisms, and naturally occurring variants. For example, homologous sequence regions can be found by sequence comparison, and equivalent enzymes can be established following the specific guidelines of the invention.

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"Functional equivalents" likewise comprise fragments, preferably individual domains or sequence motifs, of the polypeptides of the invention, which have the desired biological function, for example.

- 10 "Functional equivalents" are also fusion proteins which have one of the abovementioned polypeptide sequences or functional equivalents derived therefrom and at least one further heterologous sequence functionally different therefrom in functional N- or C-terminal linkage (i.e. with negligible functional impairment of the functions of the fusion protein parts). Nonlimiting examples of such heterologous sequences are, for example, signal peptides, enzymes,
15 immunoglobulins, surface antigens, receptors or receptor ligands.

According to the invention, "functional equivalents" include homologs of the specifically disclosed proteins. These have at least 30%, or about 40%, 50%, preferably at least about 60%, 65%, 70%, or 75%, in particular at least 85%, such as, for example, 90%, 95% or 99%, homology to
20 one of the specifically disclosed sequences, calculated by the algorithm of Pearson and Lipman, Proc. Natl. Acad., Sci. (USA) 85(8), 1988, 2444-2448.

Homologs of the proteins or polypeptides of the invention can be generated by mutagenesis, for example by point mutation or truncation of the protein. The term "homolog", as used herein,
25 relates to a variant form of the protein, which acts as agonist or antagonist of the protein activity.

Homologs of the proteins of the invention can be identified by screening combinatorial libraries of mutants such as, for example, truncation mutants. It is possible, for example, to generate a variegated library of protein variants by combinatorial mutagenesis at the nucleic acid level, for
30 example by enzymatic ligation of a mixture of synthetic oligonucleotides. There is a multiplicity of methods which can be used for preparing libraries of potential homologs from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic gene can then be ligated into a suitable expression vector. The use of a degenerate set of genes makes it possible to provide whole

sequences which encode the desired set of potential protein sequences in one mixture. Methods for synthesizing degenerate oligonucleotides are known to the skilled worker (for example, Narang, S.A., (1983) Tetrahedron 39:3; Itakura et al., (1984) Annu. Rev. Biochem. 53:323; Itakura et al., (1984) Science 198:1056; Ike et al., (1983) Nucleic Acids Res. 11:477).

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In addition, libraries of fragments of the protein codon can be used to generate a variegated population of protein fragments for screening and for subsequent selection of homologs of a protein of the invention. In one embodiment, a library of coding sequence fragments can be generated by treating a double-stranded PCR fragment of a coding sequence with a nuclease under conditions under which nicking occurs only about once per molecule, denaturing the double-stranded DNA, renaturing the DNA to form double-stranded DNA which may comprise sense/antisense pairs of various nicked products, removing single-stranded sections from newly formed duplexes by treatment with S1 nuclease and ligating the resulting fragment library into an expression vector. It is possible by this method to devise an expression library which encodes N-terminal, C-terminal and internal fragments of the protein of the invention, which has different sizes.

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Several techniques are known in the prior art for screening gene products from combinatorial libraries which have been produced by point mutations or truncation and for screening cDNA libraries for gene products with a selected property. These techniques can be adapted to rapid screening of gene libraries which have been generated by combinatorial mutagenesis of homologs of the invention. The most frequently used techniques for screening large gene libraries undergoing high-throughput analysis comprise the cloning of the gene library into replicable expression vectors, transformation of suitable cells with the resulting vector library and expression of the combinatorial genes under conditions under which detection of the desired activity facilitates isolation of the vector encoding the gene whose product has been detected. Recursive ensemble mutagenesis (REM), a technique which increases the frequency of functional mutants in the libraries, can be used in combination with the screening tests in order to identify homologs (Arkin und Yourvan (1992) PNAS 89:7811-7815; Delgrave et al. (1993) Protein Engineering 6(3):327-331

c) Polynucleotides of the invention

The invention also relates to nucleic acid sequences (single- and double-stranded DNA and

RNA sequences such as, for example cDNA and mRNA) coding for one of the above metY enzymes and the functional equivalents thereof which are obtainable, for example, also by use of artificial nucleotide analogs.

- 5 The invention relates both to isolated nucleic acid molecules which code for polypeptides or proteins of the invention or for biologically active sections thereof, and to nucleic acid fragments which can be used, for example, for use as hybridization probes or primers for identifying or amplifying coding nucleic acids of the invention.
- 10 Moreover, the nucleic acid molecules of the invention may contain untranslated sequences from the 3' and/or 5' ends of the coding region of the gene.

An "isolated" nucleic acid molecule is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid and may moreover be essentially free of other
15 cellular material or culture medium if it is prepared by recombinant techniques, or free of chemical precursors or other chemicals if it is chemically synthesized.

The invention furthermore comprises the nucleic acid molecules complementary to the specifically described nucleotide sequences or a section thereof.

20 The nucleotide sequences of the invention make it possible to generate probes and primers which can be used for identifying and/or cloning homologous sequences in other cell types and organisms. Such probes and primers usually complete a nucleotide sequence region which hybridizes under stringent conditions to at least about 12, preferably at least about 25, such as,
25 for example 40, 50 or 75, consecutive nucleotides of a sense strand of a nucleic acid sequence of the invention or of a corresponding antisense strand.

Further nucleic acid sequences of the invention are derived from SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51 or 53 and differ
30 therefrom through addition, substitution, insertion or deletion of one or more nucleotides, but still code for polypeptides having the desired profile of properties. These may be polynucleotides which are identical to above sequences in at least about 50%, 55%, 60%, 65%, 70%, 80% or 90%, preferably in at least about 95%, 96%, 97%, 98% or 99%, of the sequence positions.

The invention also includes those nucleic acid sequences which comprise "silent" mutations or are modified, by comparison with a specifically mentioned sequence, in accordance with the codon usage of a specific source or host organism, as well as naturally occurring variants such as, for example, splice variants or allelic variants. The invention likewise relates to sequences which are obtainable by conservative nucleotide substitutions (i.e. the relevant amino acid is replaced by an amino acid of the same charge, size, polarity and/or solubility).

The invention also relates to molecules derived from specifically disclosed nucleic acids through sequence polymorphisms. These genetic polymorphisms may exist because of the natural variation between individuals within a population. These natural variations usually result in a variance of from 1 to 5% in the nucleotide sequence of a gene.

The invention furthermore also comprises nucleic acid sequences which hybridize with or are complementary to the abovementioned coding sequences. These polynucleotides can be found on screening of genomic or cDNA libraries, and where appropriate, be amplified therefrom by means of PCR using suitable primers, and then, for example, be isolated with suitable probes. Another possibility is to transform suitable microorganisms with polynucleotides or vectors of the invention, multiply the microorganisms and thus the polynucleotides, and then isolate them. An additional possibility is to synthesize polynucleotides of the invention by chemical routes.

The property of being able to "hybridize" to polynucleotides means the ability of a polynucleotide or oligonucleotide to bind under stringent conditions to an almost complementary sequence, while there are no unspecific bindings between noncomplementary partners under these conditions. For this purpose, the sequences should be 70-100%, preferably 90-100%, complementary. The property of complementary sequences being able to bind specifically to one another is made use of, for example, in the Northern or Southern blot technique or in PCR or RT-PCR in the case of primer binding. Oligonucleotides with a length of 30 base pairs or more are usually employed for this purpose. Stringent conditions means, for example, in the Northern blot technique the use of a washing solution at 50-70°C, preferably 60-65°C, for example 0.1×SSC buffer with 0.1% SDS (20×SSC; 3M NaCl, 0.3M Na citrate, pH 7.0) for eluting nonspecifically hybridized cDNA probes or oligonucleotides. In this case, as mentioned above, only nucleic acids with a high degree of complementarity remain bound to one another. The setting up of stringent conditions is known to the skilled worker and is described, for example, in Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-

6.3.6.

c) Isolation of the coding metY gene

- 5 The metY genes coding for the enzyme O-acetylhomoserine sulfhydrylase can be isolated from the organisms of the above list I in a manner known per se.

In order to isolate the metY genes or else other genes of the organisms of the above list I, first a gene library of this organism is generated in Escherichia coli (E. coli). The generation of gene
10 libraries is described in detail in generally known textbooks and manuals. Examples which may be mentioned are the textbook by Winnacker: Gene und Klone, Eine Einführung in die Gentechnologie (Verlag Chemie, Weinheim, Germany, 1990), and the manual by Sambrook et al.: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989). A very well-known gene library is that of E. coli K-12 strain W3110, which was generated in λ
15 vectors by Kohara et al. (Cell 50, 495-508 (1980).

In order to produce a gene library from organisms of list I in E. coli, cosmids such as the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84: 2160-2164), or else plasmids such as pBR322 (BoliVal; Life Sciences, 25, 807-818 (1979))
20 or pUC9 (Vieira et al., 1982, Gene, 19: 259-268) can be used. Suitable hosts are in particular those E. coli strains which are restriction and recombination defective. An example of this is the strain DH5 α mcr which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids may then in turn be subcloned into common vectors suitable for sequencing and
25 subsequently be sequenced, as described, for example, in Sanger et al. (proceedings of the National Academy of Sciences of the United States of America, 74: 5463-5467, 1977).

The DNA sequences obtained can then be studied using known algorithms or sequence analysis programs such as, for example, those by Staden (Nucleic Acids Research 14, 217-232(1986)),
30 by Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program by Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

The metY-encoding DNA sequences from organisms according to the above list I were found. In particular, DNA sequences according to SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23,

25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51 and 53 were found. Furthermore, the amino acid sequences of the corresponding proteins were derived from said DNA sequences present, using the above-described methods. SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52 and 54 depict the resulting amino acid sequences of the metY gene products.

Coding DNA sequences which result from the sequences according to SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51 and 53 due to the degeneracy of the genetic code are likewise subject of the invention. In the same way, the invention relates to DNA sequences which hybridize with said sequences or parts of sequences derived therefrom.

Instructions for identifying DNA sequences by means of hybridization can be found by the skilled worker, inter alia, in the manual "The DIG System Users Guide für Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255-260). Instructions for amplifying DNA sequences with the aid of the polymerase chain reaction (PCR) can be found by the skilled worker, inter alia, in the manual by Gait: Oligonucleotide synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

It is furthermore known that changes at the N- and/or C-terminus of a protein do not substantially impair its function or may even stabilize said function. Information on this can be found by the skilled worker, inter alia, in Ben-Bassat et al. (Journal of Bacteriology 169: 751-757 (1987)), in O'Regan et al. (Gene 77: 237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3: 240-247 (1994)), in Hochuli et al. (Biotechnology 6: 1321-1325 (1988)) and in known textbooks of genetics and molecular biology.

Amino acid sequences which result accordingly from SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52 and 54 are likewise part of the invention.

d) Host cells used according to the invention

The invention further relates to microorganisms serving as host cells, in particular coryneform bacteria, which contain a vector, in particular a shuttle vector or plasmid vector, carrying at least one metY gene as defined by the invention or in which a metY gene of the invention is expressed or amplified.

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These microorganisms can produce sulfur-containing fine chemicals, in particular L-methionine, from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. Said microorganisms are preferably coryneform bacteria, in particular of the genus *Corynebacterium*. Of the genus *Corynebacterium*, mention must be made in particular of the species *Corynebacterium glutamicum* which is known in the art for its ability to produce L-amino acids.

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Examples of suitable strains of coryneform bacteria, which may be mentioned, are those of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum* (*C. glutamicum*), such as

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Corynebacterium glutamicum ATCC 13032,
Corynebacterium acetoglutamicum ATCC 15806,
Corynebacterium acetoacidophilum ATCC 13870,
Corynebacterium thermoaminogenes FERM BP-1539,
Corynebacterium melassecola ATCC 17965

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or

of the genus *Brevibacterium*, such as
Brevibacterium flavum ATCC 14067

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Brevibacterium lactofermentum ATCC 13869 and
Brevibacterium divaricatum ATCC 14020;

Or strains derived therefrom such as

Corynebacterium glutamicum KFCC10065
Corynebacterium glutamicum ATCC21608

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which likewise produce the desired fine chemical or the precursor(s) thereof.

The abbreviation KFCC means the Korean Federation of Culture Collection, the abbreviation ATCC means the American Type Strain Culture Collection, and the abbreviation FERM means the collection of the National Institute of Bioscience and Human Technology, Agency of Industrial

Science and Technology, Japan.

e) Carrying out the fermentation of the invention

- 5 According to the invention, it was found that coryneform bacteria, after overexpression of a metY gene from organisms of the list I, produce sulfur-containing fine chemicals, in particular L-methionine, in an advantageous manner.

10 To achieve overexpression, the skilled worker can take different measures individually or in combination. Thus it is possible to increase the copy number of the appropriate genes or to mutate the promoter and regulatory region or the ribosomal binding site which is located upstream of the structural gene. Expression cassettes which are incorporated upstream of the structural gene act in the same way. Inducible promoters make it additionally possible to increase expression during the course of the fermentative L-methionine production. Expression
15 is likewise improved by measures which extend the life span of the mRNA. Furthermore, the enzymic activity is likewise enhanced by preventing degradation of the enzyme protein. The genes or gene constructs may be either present in plasmids with varying copy number or integrated and amplified in the chromosome. A further possible alternative is to achieve overexpression of the relevant genes by changing the media composition and management of
20 the culture. Instructions for this can be found by the skilled worker, inter alia, in Martin et al. (Biotechnology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102, 93-98 (1991)), in the European patent 0472869, in US Patent 4,601,893, in Schwarzer and Pühler (Biotechnology 9, 84-87 (1991)), in Remscheid et al. (Applied and Environmental Microbiology 60, 126-132
25 (1994)), in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in the patent application WO 96/15246, in Malumbres et al. (Gene 134, 15-24 (1993)), in the Japanese published specification JP-A-10-229891, in Jensen und Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)), in Makrides (Microbiological Reviews 60 : 512-538 (1996)) and in known textbooks of genetics and molecular biology.

30 The invention therefore also relates to expression constructs comprising a nucleic acid sequence coding for a polypeptide of the invention under the genetic control of regulatory nucleic acid sequences; and to vectors comprising at least one of said expression constructs. Such constructs of the invention preferably include a promoter 5' upstream of the particular coding

sequence and a terminator sequence 3' downstream and also, where appropriate, further regulatory elements, in each case operatively linked to the coding sequence. An "operative linkage" means the sequential arrangement of promoter, coding sequence, terminator and, where appropriate, further regulatory elements such that each of the regulatory elements can properly carry out its function in the expression of the coding sequence. Examples of operatively linkable sequences are activating sequences and enhancers and the like. Further regulatory elements include selectable markers, amplification signals, origins of replication and the like. Suitable regulatory sequences are described, for example in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990).

In addition to the artificial regulatory sequences, the natural regulatory sequence may still be present upstream of the actual structural gene. Genetic modification can, where appropriate, switch off this natural regulation and increase or decrease expression of the genes. However, the gene construct may also have a simpler design, i.e. no additional regulatory signals are inserted upstream of the structural gene and the natural promoter with its regulation is not removed. Instead, the natural regulatory sequence is mutated such that regulation no longer takes place and gene expression is increased or reduced. The gene construct may contain one or more copies of the nucleic acid sequences.

Examples of useful promoters are: *ddh*, *amy*, *lysC*, *dapA*, *lysA* from *Corynebacterium glutamicum* promoters, but also Gram-positive promoters *SPO2*, as are described in *Bacillus Subtilis* and Its Closest Relatives, Sonenshein, Abraham L., Hoch, James A., Losick, Richard; ASM Press, District of Columbia, Washington and Patek M. Eikmanns B.J., Patek J., Sahm H., Microbiology. 142 1297-309, 1996 or else the *cos*, *tac*, *trp*, *tet*, *trp-tet*, *lpp*, *lac*, *lpp-lac*, *lacIq*, *T7*, *T5*, *T3*, *gal*, *trc*, *ara*, *SP6*, λ -PR and λ -PL promoters which are advantageously applied in Gram-negative bacteria. Preference is also given to using inducible promoters such as, for example light- and, in particular, temperature-inducible promoters such as the P_{rP_i} promoter. It is in principle possible to use all natural promoters with their regulatory sequences. In addition, it is also possible to use advantageously synthetic promoters.

The regulatory sequences mentioned are intended to make specific expression of the nucleic acid sequences possible. Depending on the host organism, this may mean, for example, that the gene is expressed or overexpressed only after induction, or that it is immediately expressed and/or overexpressed.

In this connection, the regulatory sequences and factors may preferably have a beneficial effect on, and thus increase or decrease, expression. Thus, it is possible and advantageous to enhance the regulatory elements at the transcriptional level by using strong transcription signals such as promoters and/or enhancers. However, it is also possible besides this to enhance translation by, for example, improving the stability of the mRNA.

An expression cassette is prepared by fusing a suitable promoter, a suitable Shine-Dalgarno sequence, to a metY nucleotide sequence and a suitable termination signal. For this purpose, common recombination and cloning techniques are used, such as those described, for example, in Current Protocols in Molecular Biology, 1993, John Wiley & Sons, Incorporated, New York, New York, PCR Methods, Gelfand, David H., Innis, Michael A., Sninsky, John J., 1999, Academic Press, Incorporated, California, San Diego, PCR Cloning Protocols, Methods in Molecular Biology Ser., Vol. 192, 2nd ed., Humana Press, New Jersey, Totowa. T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and in T.J. Silhavy, M.L. Berman und L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley Interscience (1987).

The recombinant nucleic acid construct or gene construct is expressed in a suitable host organism by inserting it advantageously into a host-specific vector which makes optimal expression of the genes in the host possible. Vectors are well known to the skilled worker and can be found, for example, in "Cloning Vectors" (Pouwels P. H. et al., Hrsg, Elsevier, Amsterdam-New York-Oxford, 1985). The term "vectors" means, apart from plasmids, also all other vectors known to the skilled worker, such as, for example, phages, transposons, IS elements, plasmids, cosmids and linear or circular DNA. These vectors can replicate autonomously in the host organism or are replicated chromosomally.

MetY genes of the invention were amplified by overexpressing them by way of example with the aid of episomal plasmids. Suitable plasmids are those which are replicated in coryneform bacteria. Numerous known plasmid vectors such as, for example, pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554), pEKEx1 (Eikmanns et al., Gene 102: 93-98 (1991)) or pHS2-1 (Sonnen et al., Gene 107: 69-74 (1991)) are based on the cryptic plasmids

pHM1519, pBL1 or pGA1. Other plasmid vectors such as, for example, pCLiK5MCS, or those based on pCG4 (US-A 4,489,160) or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)) or pAG1 (US-A 5,158,891) may be used in the same way.

5 Suitable plasmid vectors are furthermore also those with the aid of which it is possible to apply the method of gene amplification by integration into the chromosome, as has been described, for example, by Remscheid et al. (Applied and Environmental Microbiology 60,126-132 (1994)) for the duplication and amplification of the *hom-thrB* operon. In this method, the complete gene is cloned into a plasmid vector which can replicate in a host (typically *E. coli*) but not in *C.*
10 *glutamicum*. Suitable vectors are, for example, pSUP301 (Simon et al., Bio/ Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)), pEM1 (Schrumpf et al., 1991, Journal of Bacteriology 173: 4510-4516) or pBGS8 (Spratt et al., 1986, Gene 41: 337-342). The plasmid vector containing the gene to be amplified is then transferred into the desired *C. glutamicum*
15 strain via transformation. Methods for transformation are described, for example, in Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Biotechnology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)).

20 The activity of enzymes can be influenced by mutations in the corresponding genes in such a way that the rate of the enzymic reaction is partly or completely reduced. Examples of such mutations are known to the skilled worker (Motoyama H., Yano H., Terasaki Y., Anazawa H., Applied & Environmental Microbiology. 67:3064-70, 2001, Eikmanns BJ., Eggeling L., Sahm H., Antonie van Leeuwenhoek. 64:145-63, 1993-94.)

25 Additionally, it may be advantageous for the production of sulfur-containing fine chemicals, in particular L-methionine, to amplify, in addition to expression and amplification of a *metY* gene of the invention, one or more enzymes of the respective biosynthetic pathway, the cysteine pathway, of aspartate-semialdehyde synthesis, of glycolysis, of anaplerosis, of the pentose
30 phosphate metabolism, the citrate acid cycle or the amino acid export.

Thus, one or more of the following genes can be amplified to produce sulfur-containing fine chemicals, in particular L-methionine:

- the gene *lysC*, which encodes an aspartate kinase (EP 1 108 790 A2; DNA-SEQ NO. 281),

- the gene *asd* which encodes an aspartate-semialdehyde dehydrogenase (EP 1 108 790 A2; DNA-SEQ NO. 282),
 - the glyceraldehyde-3-phosphate dehydrogenase-encoding gene *gap* (Eikmanns (1992), Journal of Bacteriology 174: 6076-6086),
 - 5 - the 3-phosphoglycerate kinase-encoding gene *pgk* (Eikmanns (1992), Journal of Bacteriology 174: 6076-6086),
 - the pyruvate carboxylase-encoding gene *pyc* (Eikmanns (1992), Journal of Bacteriology 174: 6076-6086),
 - the triose phosphate isomerase-encoding gene *tpi* (Eikmanns (1992), Journal of Bacteriology
 - 10 174: 6076-6086),
 - the homoserine O-acetyltransferase-encoding gene *metA* (EP 1 108 790 A2; DNA-SEQ NO. 725),
 - the cystathionine gamma-synthase-encoding gene *metB* (EP 1 108 790 A2; DNA-SEQ NO. 3491),
 - 15 - the cystathionine gamma-lyase-encoding gene *metC* (EP 1 108 790 A2; DNA-SEQ NO. 3061),
 - the serine hydroxymethyltransferase-encoding gene *glyA* (EP 1 108 790 A2; DNA-SEQ NO. 1110),
 - the methionine synthase-encoding gene *metH* (EP 1 108 790 A2),
 - the methylene tetrahydrofolate reductase-encoding gene *metF* (EP 1 108 790 A2; DNA-SEQ
 - 20 NO. 2379),
 - the phosphoserine aminotransferase-encoding gene *serC* (EP 1 108 790 A2; DNA-SEQ NO. 928),
 - a phosphoserine phosphatase-encoding gene *serB* (EP 1 108 790 A2; DNA-SEQ NO. 334, DNA-SEQ NO. 467, DNA-SEQ NO. 2767),
 - 25 - the gene *cysE*, which encodes a serine acetyl transferase (EP 1 108 790 A2; DNA-SEQ NO. 2818),
 - the gene *hom*, which encodes a homoserine dehydrogenase (EP 1 108 790 A2; DNA-SEQ NO. 1306)
- 30 Thus, it may be advantageous for the production of sulfur-containing fine chemicals, in particular L-methionine, in coryneform bacteria to mutate, at the same time, at least one of the genes below, so that the activity of the corresponding proteins, compared to that of unmutated proteins, is influenced by a metabolic metabolite to a lesser extent or not at all:

- the gene *lysC*, which encodes an aspartate kinase (EP 1 108 790 A2; DNA-SEQ NO. 281),
- the pyruvate carboxylase-encoding gene *pyc* (Eikmanns (1992), Journal of Bacteriology 174: 6076-6086),
- the homoserine O-acetyltransferase-encoding gene *metA* (EP 1 108 790 A2; DNA-SEQ NO. 725),
- the cystathionine gamma-synthase-encoding gene *metB* (EP 1 108 790 A2; DNA-SEQ NO. 3491),
- the cystathionine gamma-lyase-encoding gene *metC* (EP 1 108 790 A2; DNA-SEQ NO. 3061),
- the serine hydroxymethyltransferase-encoding gene *glyA* (EP 1 108 790 A2; DNA-SEQ NO. 1110),
- the methionine synthase-encoding gene *metH* (EP 1 108 790 A2),
- the methylene tetrahydrofolate reductase-encoding gene *metF* (EP 1 108 790 A2; DNA-SEQ NO. 2379),
- the phosphoserine aminotransferase-encoding gene *serC* (EP 1 108 790 A2; DNA-SEQ NO. 928),
- a phosphoserine phosphatase-encoding gene *serB* (EP 1 108 790 A2; DNA-SEQ NO. 334, DNA-SEQ NO. 467, DNA-SEQ NO. 2767),
- the serine acetyl transferase-encoding gene *cysE* (EP 1 108 790 A2; DNA-SEQ NO. 2818),
- the gene *hom*, which encodes a homoserine dehydrogenase (EP 1 108 790 A2; DNA-SEQ NO. 1306)

It may be furthermore advantageous for the production of sulfur-containing fine chemicals, in particular L-methionine, in addition to expression and amplification of one of the *metY* genes of the invention, to attenuate one or more of the following genes, in particular to reduce expression thereof, or to switch them off:

- the homoserine kinase-encoding gene *thrB* (EP 1 108 790 A2; DNA-SEQ NO. 3453),
- the threonine dehydratase-encoding gene *ilvA* (EP 1 108 790 A2; DNA-SEQ NO. 2328),
- the threonine synthase-encoding gene *thrC* (EP 1 108 790 A2; DNA-SEQ NO. 3486),
- the meso-diaminopimelate D-dehydrogenase-encoding gene *ddh* (EP 1 108 790 A2; DNA-SEQ NO. 3494),
- the phosphoenolpyruvate carboxykinase-encoding gene *pck* (EP 1 108 790 A2; DNA-SEQ NO. 3157),
- the glucose-6-phosphate 6-isomerase-encoding gene *pgi* (EP 1 108 790 A2; DNA-SEQ NO. ...)

950),

- the pyruvate oxidase-encoding gene *poxB* (EP 1 108 790 A2; DNA-SEQ NO. 2873),
- the dihydrodipicolinate synthase-encoding gene *dapA* (EP 1 108 790 A2; DNA-SEQ NO. 3476),
- the dihydrodipicolinate reductase-encoding gene *dapB* (EP 1 108 790 A2; DNA-SEQ NO.

5 3477)

- the diaminopicolinate decarboxylase-encoding gene *lysA* (EP 1 108 790 A2; DNA-SEQ NO. 3451)

10 It may be furthermore advantageous for the production of sulfur-containing fine chemicals, in particular L-methionine, in addition to expression and amplification of one of the *metY* genes of the invention in coryneform bacteria, to mutate, at the same time, at least one of the following genes in such a way that the enzymic activity of the corresponding protein is partly or completely reduced:

- the homoserine kinase-encoding gene *thrB* (EP 1 108 790 A2; DNA-SEQ NO. 3453),
- 15 - the threonine dehydratase-encoding gene *ilvA* (EP 1 108 790 A2; DNA-SEQ NO. 2328),
- the threonine synthase-encoding gene *thrC* (EP 1 108 790 A2; DNA-SEQ NO. 3486),
- the meso-diaminopimelate D-dehydrogenase-encoding gene *ddh* (EP 1 108 790 A2; DNA-SEQ NO. 3494),

20 - the phosphoenolpyruvate carboxykinase-encoding gene *pck* (EP 1 108 790 A2; DNA-SEQ NO. 3157),

- the glucose-6-phosphate 6-isomerase-encoding gene *pgi* (EP 1 108 790 A2; DNA-SEQ NO. 950),

- the pyruvate oxidase-encoding gene *poxB* (EP 1 108 790 A2; DNA-SEQ NO. 2873),
- the dihydrodipicolinate synthase-encoding gene *dapA* (EP 1 108 790 A2; DNA-SEQ NO. 3476),

25 - the dihydrodipicolinate reductase-encoding gene *dapB* (EP 1 108 790 A2; DNA-SEQ NO. 3477)

- the diaminopicolinate decarboxylase-encoding gene *lysA* (EP 1 108 790 A2; DNA-SEQ NO. 3451)

30 It may be furthermore advantageous for the production of sulfur-containing fine chemicals, in particular L-methionine, apart from expression and amplification of a *metY* gene of the invention, to eliminate unwanted secondary reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention may be cultured continuously or batchwise or in a fed batch or repeated fed batch process to produce sulfur-containing fine chemicals, in particular L-methionine. An overview of known cultivation methods can be found
5 in the textbook by Chmiel (Bioprozeßtechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must satisfy the demands of the particular strains in a suitable
10 manner. The textbook "Manual of Methods für General Bacteriology" by the American Society for Bacteriology (Washington D. C., USA, 1981) contains descriptions of culture media for various microorganisms.

Said media which can be used according to the invention usually comprise one or more carbon
15 sources, nitrogen sources, inorganic salts, vitamins and/or trace elements.

Preferred carbon sources are sugars such as mono-, di- or polysaccharides. Examples of very good carbon sources are glucose, fructose, mannose, galactose, ribose, sorbose, ribulose, lactose, maltose, sucrose, raffinose, starch and cellulose. Sugars may also be added to the
20 media via complex compounds such as molasses or other byproducts of sugar refining. It may also be advantageous to add mixtures of different carbon sources. Other possible carbon sources are oils and fats such as, for example, soybean oil, sunflower oil, peanut oil and coconut fat, fatty acids such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols such as, for example, glycerol, methanol and ethanol and organic acids such as, for example acetic
25 acid and lactic acid.

Nitrogen sources are usually organic or inorganic hydrogen compounds or materials containing said compounds. Examples of nitrogen sources include ammonia gas or ammonium salts such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and
30 ammonium nitrate, nitrates, urea, amino acids and complex nitrogen sources such as cornsteep liquor, soybean flour, soybean protein, yeast extract, meat extract and others. The nitrogen sources may be used singly or as mixture.

Inorganic salt compounds which may be included in the media comprise the chloride,

phosphorus or sulfate salts of calcium, magnesium, sodium, cobalt, molybdenum, potassium, manganese, zinc, copper and iron.

5 Inorganic sulfur-containing compounds such as, for example, sulfates, sulfites, dithionites, tetrathionates, thiosulfates, sulfides, or else organic sulfur compounds such as mercaptans and thiols may be used as sources of sulfur for the production of sulfur-containing fine chemicals, in particular of methionine.

10 Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts may be used as sources of phosphorus.

15 Chelating agents may be added to the medium in order to keep the metal ions in solution. Particularly suitable chelating agents include dihydroxyphenols such as catechol or protocatechuate and organic acids such as citric acid.

20 The fermentation media used according to the invention usually also contain other growth factors such as vitamins or growth promoters, which include, for example, biotin, riboflavin, thiamine, folic acid, nicotinic acid, panthothenate and pyridoxine. Growth factors and salts are frequently derived from complex media components such as yeast extract, molasses, cornsteep liquor and the like. It is moreover possible to add suitable precursors to the culture medium. The exact composition of the media heavily depends on the particular experiment and is decided upon individually for each specific case. Information on the optimization of media can be found in the textbook "Applied Microbiol. Physiology, A Practical Approach" (Editors. P.M. Rhodes, P.F. Stanbury, IRL Press (1997) pp. 53-73, ISBN 0 19 963577 3). Growth media can also be obtained
25 from commercial suppliers, for example Standard 1 (Merck) or BHI (brain heart infusion, DIFCO) and the like.

30 All media components are sterilized, either by heat (20 min at 1.5 bar and 121°C) or by sterile filtration. The components may be sterilized either together or, if required, separately. All media components may be present at the start of the cultivation or added continuously or batchwise, as desired.

The culture temperature is normally between 15°C and 45°C, preferably at from 25°C to 40°C, and may be kept constant or may be altered during the experiment. The pH of the medium

should be in the range from 5 to 8.5, preferably around 7.0. The pH for cultivation can be controlled during cultivation by adding basic compounds such as sodium hydroxide, potassium hydroxide, ammonia and aqueous ammonia or acidic compounds such as phosphoric acid or sulfuric acid. Foaming can be controlled by employing antifoams such as, for example, fatty acid polyglycol esters. To maintain the stability of plasmids it is possible to add to the medium suitable substances having a selective effect, for example antibiotics. Aerobic conditions are maintained by introducing oxygen or oxygen-containing gas mixtures such as, for example, air into the culture. The temperature of the culture is normally 20°C to 45°C. The culture is continued until formation of the desired product is at a maximum. This aim is normally achieved within 10 to 160 hours.

The fermentation broths obtained in this way, in particular those containing L-methionine, usually contain a dry biomass of from 7.5 to 25% by weight.

- 15 An additional advantage is to carry out the fermentation under sugar limitation, at least at the end, but in particular over at least 30% of the fermentation period. This means that during this time the concentration of utilizable sugar in the fermentation medium is maintained at or reduced to ≥ 0 to 3 g/l.
- 20 The fermentation broth is then processed further. The biomass may, according to requirement, be removed completely or partially from the fermentation broth by separation methods such as, for example, centrifugation, filtration, decanting or a combination of these methods or be left completely in said broth.
- 25 Subsequently, the fermentation broth may be thickened or concentrated using known methods such as, for example, with the aid of a rotary evaporator, thin film evaporator, falling film evaporator, by reverse osmosis, or by nanofiltration. This concentrated fermentation broth can then be worked up by freeze drying, spray drying, spray granulation or by other methods.
- 30 However, it is also possible to further purify the sulfur-containing fine chemicals, in particular L-methionine. To this end, the product-containing broth, after removing the biomass, is subjected to a chromatography using a suitable resin, the desired product or the contaminations being retained completely or partially on the chromatographic resin. These chromatographic steps can be repeated, if necessary, using the same or different chromatographic resin. The skilled worker

is familiar with the selection of suitable chromatographic resins and their most effective application. The purified product can be concentrated by filtration or ultrafiltration and stored at a temperature at which the stability of the product is greatest.

- 5 The identity and purity of the isolated compound(s) can be determined by techniques of the art. These include high performance liquid chromatography (HPLC), spectroscopic methods, staining methods, thin-layer chromatography, NIRS, enzyme assay or microbiological assays. These analytic methods are summarized in: Patek et al. (1994) Appl. Environ. Microbiol. 60:133-140; Malakhova et al. (1996) Biotekhnologiya 11 27-32; and Schmidt et al. (1998) Bioprocess
10 Engineer. 19:67-70. Ulmann's Encyclopedia of Industrial Chemistry (1996) Bd. A27, VCH: Weinheim, pp. 89-90, pp. 521-540, pp. 540-547, pp. 559-566, 575-581 and pp. 581-587; Michal, G., (1999) Biochemical Pathways: An Atlas of Biochemistry and Molecular Biology, John Wiley and Sons; Fallon, A. et al. (1987) Applications of HPLC in Biochemistry in: Laboratory Techniques in Biochemistry and Molecular Biology, Volume 17.

15

The invention is now described in greater detail with reference to the following nonlimiting examples and with reference to the appended figures, in which

- 20 Figure 1 shows the plasmid map for plasmid pClysC;
Figure 2 the plasmid map for plasmid pCISlysCthr311ile;
Figure 3 the plasmid map for plasmid pCPhsdhmetY_Mt.
Restriction cleavage sites with the corresponding indication of their position in brackets are shown in the plasmid maps. Essential sequence segments are described in bold. KanR means
25 kanamycin resistance gene; ask means aspartate kinase gene.

Example 1: Construction of pCLiK5MCS

- First, ampicillin resistance and origin of replication of the vector pBR322 were amplified using
30 the oligonucleotides p1.3 (SEQ ID NO:55) and p2.3 (SEQ ID NO:56) with the aid of the polymerase chain reaction (PCR).

p1.3 (SEQ ID NO:55)

5'-CCCGGGATCCGCTAGCGGCGCGCCGGCCGGCCCGGTGTGAAATACCGCACAG-3'

p2.3 (SEQ ID NO:56)

5 5'-TCTAGACTCGAGCGGCCGCGGCCGGCCTTTAAATTGAAGACGAAAGGGCCTCG-3'

10 In addition to sequences complementary to pBR322, the oligonucleotide p1.3 (SEQ ID NO:55) contains in 5'-3' direction the cleavage sites for the restriction nucleases SmaI, BamHI, NheI and Ascl and the oligonucleotide p2.3 (SEQ ID NO:56) contains in 5'-3' direction the cleavage sites
15 for the restriction endonucleases XbaI, XhoI, NotI and DraI. The PCR reaction was carried out according to a standard method such as that by Innis et al. (PCR Protocols. A Guide to Methods and Applications, Academic Press (1990)) using PfuTurbo polymerase (Stratagene, La Jolla, USA). The DNA fragment obtained of approximately 2.1 kb in size was purified using the
20 GFX™PCR, DNA and gel band purification kit (Amersham Pharmacia, Freiburg) according to the manufacturer's instructions. The blunt ends of the DNA fragment were ligated to one another using the rapid DNA ligation kit (Roche Diagnostics, Mannheim) according to the manufacturer's instructions and the ligation mixture was transformed into competent E. coli XL-1Blue (Stratagene, La Jolla, USA) according to standard methods, as described in Sambrook et al. (Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, (1989)). Plasmid-carrying cells
25 were selected for by plating out onto ampicillin (50 µg/ml)-containing LB agar (Lennox, 1955, Virology, 1:190).

The plasmid DNA of an individual clone was isolated using the Qiaprep spin miniprep kit (Qiagen, Hilden) according to the manufacturer's instructions and checked by restriction digests.
25 The plasmid obtained in this way is denoted pCLiK1.

Starting from plasmid pWLT1 (Liebl et al., 1992) as template for a PCR reaction, a kanamycin resistance cassette was amplified using the oligonucleotides neo1 (SEQ ID NO:57) and neo2 (SEQ ID NO:58).

30

neo1 (SEQ ID NO:57):

5'-GAGATCTAGACCCGGGGATCCGCTAGCGGGCTGCTAAAGGAAGCGGA-3'

neo2 (SEQ ID NO:58):

5'-GAGAGGCGCGCCGCTAGCGTGGGCGAAGAACTCCAGCA-3'

Apart from the sequences complementary to pWLT1, the oligonucleotide neo1 contains in 5'-3' direction the cleavage sites for the restriction endonucleases XbaI, SmaI, BamHI, NheI and the oligonucleotide neo2 (SEQ ID NO:58) contains in 5'-3' direction the cleavage sites for the restriction endonucleases AscI and NheI. The PCR reaction was carried out using PfuTurbo polymerase (Stratagene, La Jolla, USA) according to a standard method such as that of Innis et al. (PCR Protocols. A Guide to Methods and Applications, Academic Press (1990)). The DNA fragment obtained was approximately 1.3 kb in size was purified using the GFXTMPCR, DNA and gel band purification kit (Amersham Pharmacia, Freiburg) according to the manufacturer's instructions. The DNA fragment was cleaved with restriction endonucleases XbaI and AscI (New England Biolabs, Beverly, USA) and, following that, again purified using the GFXTMPCR, DNA and gel band purification kit (Amersham Pharmacia, Freiburg) according to the manufacturer's instructions. The vector pCLiK1 was likewise cleaved with the restriction endonucleases XbaI and AscI and dephosphorylated using alkaline phosphatase (Roche Diagnostics, Mannheim) according to the manufacturer's instructions. After electrophoresis in a 0.8% strength agarose gel, the linearized vector (approx. 2.1 kb) was isolated using the GFXTMPCR, DNA and gel band purification kit (Amersham Pharmacia, Freiburg) according to the manufacturer's instructions. This vector fragment was ligated with the cleaved PCR fragment with the aid of the rapid DNA ligation kit (Roche Diagnostics, Mannheim) according to the manufacturer's instructions and the ligation mixture was transformed into competent E. coli XL-1Blue (Stratagene, La Jolla, USA) according to standard methods, as described in Sambrook et al. (Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, (1989)). Plasmid-carrying cells were selected for by plating out onto ampicillin (50 µg/ml)- and kanamycin (20 µg/ml)-containing LB agar (Lennox, 1955, Virology, 1:190).

The plasmid DNA of an individual clone was isolated using the Qiaprep spin miniprep kit (Qiagen, Hilden) according to the manufacturer's instructions and checked by restriction digests. The plasmid obtained in this way is denoted pCLiK2.

The vector pCLiK2 was cleaved with the restriction endonuclease DraI (New England Biolabs, Beverly, USA). After electrophoresis in 0.8% strength agarose gel, an approx. 2.3 kb vector

fragment was isolated using the GFXTMPCR, DNA and gel band purification kit (Amersham Pharmacia, Freiburg) according to the manufacturer's instructions. This vector fragment was religated with the aid of the rapid DNA ligation kit (Roche Diagnostics, Mannheim) according to the manufacturer's instructions and the ligation mixture was transformed into competent *E. coli* XL-1Blue (Stratagene, La Jolla, USA) according to standard methods, as described in Sambrook et al. (Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, (1989)). Plasmid-carrying cells were selected for by plating out onto kanamycin (20 µg/ml)-containing LB agar (Lennox, 1955, Virology, 1:190).

- 10 The plasmid DNA of an individual clone was isolated using the Qiaprep spin miniprep kit (Qiagen, Hilden) according to the manufacturer's instructions and checked by restriction digests. The plasmid obtained in this way is denoted pCLiK3.

15 Starting from plasmid pWLQ2 (Liebl et al., 1992) as template for a PCR reaction, the origin of replication pHM1519 was amplified using the oligonucleotides cg1 (SEQ ID NO:59) and cg2 (SEQ ID NO:60).

cg1 (SEQ ID NO:59):

5'-GAGAGGGCGGCCGCGCAAAGTCCCGCTTCGTGAA-3'

20

cg2 (SEQ ID NO:60):

5'-GAGAGGGCGGCCGCTCAAGTCGGTCAAGCCACGC-3'

- 25 Apart from the sequences complementary to pWLQ2, the oligonucleotides cg1 (SEQ ID NO:59) and cg2 (SEQ ID NO:60) contain cleavage sites for the restriction endonuclease NotI. The PCR reaction was carried out using PfuTurbo polymerase (Stratagene, La Jolla, USA) according to a standard method such as that of Innis et al. (PCR Protocols. A Guide to Methods and Applications, Academic Press (1990)). The DNA fragment obtained was approximately 2.7 kb in size and was purified using the GFXTMPCR, DNA and gel band purification kit (Amersham Pharmacia, Freiburg) according to the manufacturer's instructions. The DNA fragment was
- 30 cleaved with restriction endonuclease NotI (New England Biolabs, Beverly, USA) and, following that, again purified using the GFXTMPCR, DNA and gel band purification kit (Amersham Pharmacia, Freiburg) according to the manufacturer's instructions. The vector pCLiK3 was likewise cleaved with the restriction endonuclease NotI and dephosphorylated using alkaline

phosphatase (Roche Diagnostics, Mannheim) according to the manufacturer's instructions. After electrophoresis in a 0.8% strength agarose gel, the linearized vector (approx. 2.3 kb) was isolated using the GFX™PCR, DNA and gel band purification kit (Amersham Pharmacia, Freiburg) according to the manufacturer's instructions. This vector fragment was ligated with the cleaved PCR fragment with the aid of the rapid DNA ligation kit (Roche Diagnostics, Mannheim) according to the manufacturer's instructions and the ligation mixture was transformed into competent *E. coli* XL-1Blue (Stratagene, La Jolla, USA) according to standard methods, as described in Sambrook et al. (Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, (1989)). Plasmid-carrying cells were selected for by plating out onto kanamycin (20 µg/ml)-containing LB agar (Lennox, 1955, Virology, 1:190).

The plasmid DNA of an individual clone was isolated using the Qiaprep spin miniprep kit (Qiagen, Hilden) according to the manufacturer's instructions and checked by restriction digests. The plasmid obtained in this way is denoted pCLiK5.

PCLiK5 was extended by a multiple cloning site (MCS) by combining the two synthetic essentially complementary oligonucleotides HS445 ((SEQ ID NO:61) and HS446 (SEQ ID NO:62)) which contain cleavage sites for the restriction endonucleases *Swa*I, *Xho*I, *Aat*I, *Ap*aI, *Asp*718, *Mlu*I, *Nde*I, *Spe*I, *Eco*RV, *Sal*I, *Cl*aI, *Bam*HI, *Xba*I and *Sma*I to give a double-stranded DNA fragment by heating them together to 95°C followed by slow cooling.

HS445 (SEQ ID NO:61):

5'-TCGAATTTAAATCTCGAGAGGCCTGACGTCGGGCCCCGGTACCACGCGTCATATGACTAG
TTCGGACCTAGGGATATCGTCGACATCGATGCTCTTCTGCGTTAATTAACAATTGGGATCC
TCTAGACCCGGGATTAAAT-3'

HS446 (SEQ ID NO:62):

5'-GATCATTTAAATCCCGGGTCTAGAGGATCCCAATTGTAAATTAACGCAGAAGAGCATCGA
TGTCGACGATATCCCTAGGTCCGAAGTAGTCATATGACGCGTGGTACCGGGCCCCGACGTC
AGGCCTCTCGAGATTAAAT-3'

The vector pCLiK5 was cleaved with the restriction endonucleases *Xho*I and *Bam*HI (New England Biolabs, Beverly, USA) and dephosphorylated using alkaline phosphatase (I (Roche Diagnostics, Mannheim)) according to the manufacturer's instructions. After electrophoresis in

a 0.8% strength agarose gel, the linearized vector (approx. 5.0 kb) was isolated using the GFX™PCR, DNA and gel band purification kit (Amersham Pharmacia, Freiburg) according to the manufacturer's instructions. This vector fragment was ligated with the synthetic double-stranded DNA fragment with the aid of the rapid DNA ligation kit (Roche Diagnostics, Mannheim) according to the manufacturer's instructions and the ligation mixture was transformed into competent *E. coli* XL-1Blue (Stratagene, La Jolla, USA) according to standard methods as described Sambrook et al. (Molecular Cloning. A Laboratory Manual, Cold Spring Harbor (1989)). Plasmid-carrying cells were selected for by plating out onto kanamycin (20 µg/ml)-containing LB agar (Lennox, 1955, Virology, 1:190).

The plasmid DNA of an individual clone was isolated using the Qiaprep spin miniprep kit (Qiagen, Hilden) according to the manufacturer's instructions and checked by restriction digests. The plasmid obtained in this way is denoted pCLiK5MCS.

Sequencing reactions were carried out according to Sanger et al. (1977) Proceedings of the National Academy of Sciences USA 74:5463-5467. The sequencing reactions were fractionated and analyzed by means of ABI Prism 377 (PE Applied Biosystems, Weiterstadt).

The resultant plasmid pCLiK5MCS is listed as SEQ ID NO: 65.

Example 2: Construction of pCLiK5MCS integrativ sacB

Starting from the plasmid pK19mob (Schäfer et al., Gene 145,69-73(1994)) as template for a PCR reaction, the *Bacillus subtilis* sacB gene (coding for levan sucrase) was amplified using the oligonucleotides BK1732 and BK1733.

BK1732 (SEQ ID NO:63):

5'-GAGAGCGGCCGCGGATCCTTTTAAACCCATCAC-3'

BK1733 (SEQ ID NO:64):

5'-AGGAGCGGCCGCGCATCGGCATTTTCTTTTGCG-3'

Apart from the sequences complementary to pEK19mobsac, the oligonucleotides BK1732 and BK1733 contain cleavage sites for the restriction endonuclease NotI. The PCR reaction was

carried out using PfuTurbo polymerase (Stratagene, La Jolla, USA) using a standard method like that of Innis et al. (PCR Protocols. A Guide to Methods and Applications, Academic Press (1990)). The DNA fragment obtained of approximately 1.9 kb in size was purified using the GFX™PCR, DNA and gel band purification kit (Amersham Pharmacia, Freiburg) according to the manufacturer's instructions. The DNA fragment was cleaved with the restriction endonuclease NotI (New England Biolabs, Beverly, USA) and, following that, again purified using the GFX™PCR, DNA and gel band purification kit (Amersham Pharmacia, Freiburg) according to the manufacturer's instructions.

- 10 The vector pCLiK5MCS (prepared according to example 1) was likewise cleaved with the restriction endonuclease NotI and dephosphorylated using alkali phosphatase (I (Roche Diagnostics, Mannheim)) according to the manufacturer's instructions. After electrophoresis in a 0.8% strength agarose gel, an approximately 2.4 kb in size vector fragment was isolated using the GFX™PCR, DNA and gel band purification kit (Amersham Pharmacia, Freiburg) according to the manufacturer's instructions. This vector fragment was ligated with the cleaved PCR fragment with the aid of the rapid DNA ligation kit (Roche Diagnostics, Mannheim) according to the manufacturer's instructions and the ligation mixture was transformed into competent E. coli XL-1Blue (Stratagene, La Jolla, USA) according to standard methods, as described in Sambrook et al. (Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, (1989)). Plasmid-carrying cells were selected for by plating out onto kanamycin (20 µg/ml)-containing LB agar (Lennox, 1955, Virology, 1:190).

The plasmid DNA of an individual clone was isolated using the Qiaprep spin miniprep kit (Qiagen, Hilden) according to the manufacturer's instructions and checked by restriction digests.

- 25 The plasmid obtained in this way is denoted pCLiK5MCS integrativ sacB.

Sequencing reactions were carried out according to Sanger et al. (1977) Proceedings of the National Academy of Sciences USA 74:5463-5467. The sequencing reactions were fractionated and analyzed by means of ABI Prism 377 (PE Applied Biosystems, Weiterstadt).

30

The resultant plasmid pCLiK5MCS integrativ sacB is listed as SEQ ID NO: 66.

It is possible to prepare in an analog manner further vectors which are suitable for the inventive expression or overproduction of metY genes.

Example 3: Isolation of the *lysC* gene from the *C. glutamicum* strain LU1479

In the first step of the strain construction, it is intended to carry out an allelic substitution of the *lysC* wild-type gene encoding the enzyme aspartate kinase in *C. glutamicum* ATCC13032, hereinbelow referred to as LU1479. It is intended to carry out a nucleotide substitution in the *LysC* gene so that the amino acid Ile is substituted for the amino acid Thr at position 311 in the resulting protein.

Starting from the chromosomal DNA of LU1479 as template for a PCR reaction, an amplification was carried out with the oligonucleotide primers SEQ ID NO:67 and SEQ ID NO:68 *lysC* with the aid of the Pfu-Turbo PCR system (Stratagene USA) following the manufacture's instructions. Chromosomal DNA from *C. glutamicum* ATCC 13032 was prepared as described by Tauch et al. (1995) Plasmid 33:168-179 or Eikmanns et al. (1994) Microbiology 140:1817-1828. The amplified fragment is flanked at its 5' end by a *Sall* restriction cleavage and at its 3' end by an *Mlul* restriction cleavage. Prior to the cloning step, the amplified fragment was restricted by these two restriction enzymes and purified using GFX™PCR, DNA and gel band purification kit (Amersham Pharmacia, Freiburg).

SEQ ID NO:67

5'-GAGAGAGAGACGCGTCCCAGTGGCTGAGACGCATC -3'

SEQ ID NO:68

5'-CTCTCTCTGTGCGACGAATTCAATCTTACGGCCTG-3'

The resulting polynucleotide was cloned into pCLIK5 MCS integrativ *SacB* (hereinbelow referred to as pCIS; SEQ ID NO: 66 of Example 2) via the *Sall* and *Mlul* restriction cleavages and transformed into *E.coli* XL-1 blue. A selection for plasmid-bearing cells was achieved by plating onto kanamycin (20µg/ml)-containing LB agar (Lennox, 1955, Virology, 1:190). The plasmid was isolated and the expected nucleotide sequence was verified by sequencing. The plasmid DNA preparation was carried out by methods of, and using material from, Qiagen. Sequencing reactions were carried out as described by Sanger et al. (1977) Proceedings of the National Academy of Sciences USA 74:5463-5467. The sequencing reactions were separated by means of ABI Prism 377 (PE Applied Biosystems, Weiterstadt) and evaluated. The resulting plasmid pCIS *lysC* is shown as SEQ ID NO:69. The corresponding plasmid map is shown in Figure 1.

The sequence SEQ ID NO:69 encompasses the following essential part-regions:

LOCUS pCIS\lysC 5860 bp DNA circular

FEATURES Location/Qualifiers

```

5   CDS1)      155..1420
           /vntifkey="4"
           /label=lysC
           CDS      complement2)(3935..5356)
           /vntifkey="4"
10          /label=sacB\Bacillus\subtilis
           promoter complement(5357..5819)
           /vntifkey="30"
           /label=Promotor\sacB
           C_region complement(3913..3934)
15          /vntifkey="2"
           /label=sacB\downstream region
           CDS      1974..2765
           /vntifkey="4"
           /label=Kan\R
20          CDS      complement(3032..3892)
           /vntifkey="4"
           /label=Ori\EC\pMB)

```

¹⁾ coding sequence

25 ²⁾ on the complementary strand

Example 4: Mutagenesis of the *C. glutamicum* lysC gene

The site-directed mutagenesis of the *C. glutamicum* lysC gene (Example 3) was carried out with the QuickChange Kit (Stratagene/USA) following the manufacturer's instructions. The mutagenesis was carried out in the plasmid pCIS lysC, SEQ ID NO:69. The following oligonucleotide primers were synthesized for the substitution of thr311 for 311ile with the aid of the Quickchange method (Stratagene):

SEQ ID NO:70

5'-CGGCACCACCGACATCATCTTCACCTGCCCTCGTTCCG-3'

SEQ ID NO:71

5'-CGGAACGAGGGCAGGTGAAGATGATGTCGGTGGTGCCG -3'

- 5 The use of these oligonucleotide primers in the Quickchange reaction brings about a substitution of the nucleotide in position 932 (T for C) in the *lysC* gene (cf. SEQ ID NO:72) and an amino acid substitution in position 311 (Thr→Ile) (cf. SEQ ID NO:73) in the corresponding enzyme. The resulting amino acid substitution Thr311Ile in the *lysC* gene was confirmed by sequencing following transformation into E.coli XL1-blue and plasmid preparation. The plasmid was named
- 10 pCIS *lysC* thr311ile and is listed as SEQ ID NO:74. The corresponding plasmid map is shown in Figure 2.

The sequence SEQ ID NO:74 encompasses the following essential part-regions:

15 LOCUS pCIS\lysC\thr311ile 5860 bp DNA circular
 FEATURES Location/Qualifiers
 CDS¹⁾ 155..1420
 /vntifkey="4"
 /label=lysC
 20 CDS complement²⁾(3935..5356)
 /vntifkey="4"
 /label=sacB\Bacillus\subtilis
 promoter complement(5357..5819)
 /vntifkey="30"
 25 /label=Promotor\sacB
 C_region complement(3913..3934)
 /vntifkey="2"
 /label=sacB\downstream region
 CDS 1974..2765
 /vntifkey="4"
 30 /label=Kan\R
 CDS complement(3032..3892)
 /vntifkey="4"
 /label=Ori\EC\pMB)

35

¹⁾ coding sequence

²⁾ on the complementary strand

The plasmid pCIS lysC thr311ile was transformed into *C. glutamicum* LU1479 by means of electroporation as described by Liebl, et al. (1989) FEMS Microbiology Letters 53:299-303. Modifications of the protocol are described in DE-A-10046870. The chromosomal arrangement of the lysC locus of individual transformants was verified using standard methods by means of Southern blotting and hybridization as described in Sambrook et al. (1989), Molecular Cloning. A Laboratory Manual, Cold Spring Harbor. It was thus ensured that the transformants were transformants which have the transformed plasmid integrated at the lysC locus by homologous recombination. After such colonies were grown overnight in media without antibiotic, the cells were plated onto a sucrose CM agar medium (10% sucrose) and incubated for 24 hours at 30°C.

Since the sacB gene which is present in the vector pCIS lysC thr311ile converts sucrose into a toxic product, only those colonies are capable of growing which have the sacB gene deleted between the wild-type lysC gene and the mutated gene lysC thr311ile by a second homologous recombination step. During the homologous recombination, either the wild-type gene or the mutated gene can be deleted together with the sacB gene. If the sacB gene is removed together with the wild-type gene, the result is a mutated transformant.

Growing colonies were picked and examined for a kanamycin-sensitive phenotype. Clones with deleted SacB gene must simultaneously show kanamycin-sensitive growth behavior. Such Kan-sensitive clones were examined for their lysine productivity in a shaker flask (see Example 6). For comparison, the untreated strain LU1479 was cultured. Clones whose lysine production was increased in comparison with the control were selected, chromosomal DNA was obtained, and the corresponding region of the lysC gene was amplified by a PCR reaction and sequenced. Such a clone with the characteristic of an increased lysine synthesis and a confirmed mutation in lysC at position 932 was termed LU1479 lysC 311ile).

Example 5: Generation of ethionine-resistant *C. Glutamicum* strains

In the second step of the strain construction, the resulting strain LU1479 lysC 311ile (Example 4) was treated in order to induce ethionine resistance (Kase, H. Nakayama K.Agr. Biol. Chem. 39 153-106 1975 L-methionine production by methionine analog-resistant mutants of *Corynebacterium glutamicum*). An overnight culture in BHI medium (Difco) was washed in citrate buffer (50mM pH 5.5) and treated for 20 minutes at 30°C with N-methylnitrosoguanidine (10mg/ml in 50mM citrate pH5.5). After treatment with the chemical mutagen N-methyl-

nitrosoguanidine, the cells were washed (citrate buffer 50mM pH 5.5) and plated onto a medium composed of the following components, based on 500ml: 10g (NH₄)₂SO₄, 0.5g KH₂PO₄, 0.5g K₂HPO₄, 0.125g MgSO₄·7H₂O, 21g MOPS, 50mg CaCl₂, 15mg proteocatechuate, 0.5mg biotin, 1mg thiamine, 5g/l D,L-ethionine (Sigma Chemicals Deutschland), pH 7.0. The medium additionally comprised 0.5ml of a microsalt solution of: 10g/l FeSO₄·7H₂O, 1g/l MnSO₄·H₂O, 0.1g/l ZnSO₄·7H₂O, 0.02g/l CuSO₄, 0.002g/l NiCl₂·6H₂O. All salts were dissolved in 0.1M HCl. The finished medium was filter-sterilized and, after addition of 40 ml sterile 50% glucose solution, treated with liquid sterile agar in a final concentration of 1.5% agar and poured into culture dishes.

Mutagen-treated cells were placed onto plates containing the above-described medium and incubated for 3-7 days at 30°C. Resulting clones were isolated, isolated individually at least once on the selection medium and then examined for their methionine productivity in medium II in a shake flask (see Example 6).

Example 6: Methionine production with strain LU1479 lysC 311ile ET-16.

The strains produced in Example 5 were grown for 2 days at 30°C on an agar plate with CM medium.

CM agar:

10.0 g/l D-glucose, 2.5 g/l NaCl, 2.0 g/l urea, 10.0 g/l Bacto peptone (Difco), 5.0 g/l yeast extract (Difco), 5.0 g/l beef extract (Difco), 22.0 g/l agar (Difco), autoclaved (20 min., 121°C)

The cells were subsequently scraped off the plate and resuspended in saline. For the main culture, 10 ml of medium II and 0.5 g autoclaved CaCO₃ (Riedel de Haen) in a 100 ml Erlenmeyer flask were inoculated with the cell suspension until an OD_{600nm} of 1.5 was reached and incubated for 72 hours at 30°C on an orbital shaker at 200 rpm.

Medium II:

40g/l	Sucrose
60g/l	Molasses (based on 100% sugar content)
10g/l	(NH ₄) ₂ SO ₄
0.4g/l	MgSO ₄ ·7H ₂ O

0.6g/l KH_2PO_4
 0.3mg/l Thiamine*HCl
 1mg/l Biotin (from a 1 mg/ml filter-sterilized stock solution which had been brought to pH 8.0 with NH_4OH)
 5 2mg/l FeSO_4
 2mg/l MnSO_4

brought to pH 7.8 with NH_4OH , autoclaved (121°C , 20 min). In addition, vitamin B12 (hydroxycobalamine Sigma Chemicals) from a stock solution (200 $\mu\text{g/ml}$, filter-sterilized) was added to a final concentration of 100 $\mu\text{g/l}$.

10

The methionine formed, and other amino acids in the culture broth, were [lacuna] with the aid of the amino acid acid determination method from Agilent using a Agilent 1100 Series LC System HPLC. Derivatization before the column separation with ortho-phthalaldehyde enabled the quantification of the amino acids formed. The amino acid mixture was separated on a
 15 Hypersil AA column (Agilent).

Those clones whose methionine productivity was at least twice as high as that of the original strain LU1479 lysC 311ile were isolated. Such a clone was employed for the further experiments and was named LU1479 lysC 311ile ET-16.

20

Example 7: Cloning metY from *Mycobacterium tuberculosis* and cloning into the plasmid pC Phsdh metY_Mt

25

Chromosomal DNA of *Mycobacterium tuberculosis* was obtained from the American Type Strain Culture Collection (ATCC, Atlanta-USA) from strain ATCC 25584. Chromosomal DNA from *C. glutamicum* ATCC 13032 was prepared by the method described by Tauch et al. (1995) Plasmid 33:168-179 or Eikmanns et al. (1994) Microbiology 140:1817-1828.

30

Using the oligonucleotide primers SEQ ID NO:75 and SEQ ID NO:76, the chromosomal DNA from *C. glutamicum* as template and Pfu Turbo polymerase (Stratagene), an approx. 180 base pair DNA fragment was amplified from the noncoding 5' region (promoter region) of homoserine dehydrogenase (HsDH) with the aid of the polymerase chain reaction (PCR) following standard methods, such as Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press. The amplified fragment is flanked at its 5' end by a BamHI restriction cleavage

site and at its 3' end by a region which is homologous to metY from *Mycobacterium tuberculosis* and has been introduced via the oligo.

SEQ ID NO:75

5 5'-GAGAGGATCCGGAAGGTGAATCGAATTTTCGG -3'

and

SEQ ID NO:76

5'-CTATTGCTGTCTGGCGCTCATGATTCTCCAAAATAATCGC -3'

- 10 The resulting DNA fragment was purified using the GFX™PCR, DNA and gel band purification kit (Amersham Pharmacia, Freiburg) following the manufacturer's instructions.

Starting from the chromosomal DNA from *Mycobacterium tuberculosis* as template for a PCR reaction, metY was amplified with the aid of the GC-rich PCR system (Roche Diagnostics, Mannheim) following the manufacturer's instructions, using the oligonucleotide primers SEQ ID NO:77 and SEQ ID NO:78. The amplified fragment is flanked at its 3' end by an XbaI restriction cleavage site which has been introduced via the oligo.

SEQ ID NO:77

20 5'-ATGAGCGCCGACAGCAATAG -3'

and

SEQ ID NO:78

5'-GAACTCTAGATCAGAACGCCGCCACGGAC -3'

- 25 The approximately 1.4 kb DNA fragment which was obtained was purified with the GFX™PCR, DNA and gel band purification kit (Amersham Pharmacia, Freiburg) following the manufacturer's instructions.

In a further PCR reaction, the two fragments obtained above were employed jointly as template.

- 30 Owing to the regions which are homologous to the metY fragment and which have been introduced with the oligonucleotide primer SEQ ID NO:76, the two fragments anneal with one another during the PCR reaction and are elongated to a continuous DNA strand by the polymerase employed. The standard method was modified insofar as the oligonucleotide primers used, SEQ ID NO:75 and SEQ ID NO:78, were only added to the reaction at the beginning of

the 2nd cycle.

The amplified DNA fragment, which was approximately 1.6 kb in size, was purified with the GFX™PCR, DNA and gel band purification kit following the manufacturer's instructions.

5 Thereafter, it was cleaved with the restriction enzymes BamHI and XbaI (Roche Diagnostics, Mannheim) and separated by gel electrophoresis. The approximately 1.6 kb DNA fragment was subsequently isolated from the agarose using the GFX™PCR, DNA and gel band purification kit (Amersham Pharmacia, Freiburg).

10 The vector pClik5MCS SEQ ID NO:65, hereinbelow referred to as pC, was cleaved with the restriction enzymes BamHI and XbaI (Roche Diagnostics, Mannheim), and, after separation by electrophoresis, a 5 kb fragment was isolated with the GFX™PCR, DNA and gel band purification kit.

15 The vector fragment together with the cleaved and isolated PCR fragment were ligated with the aid of the Rapid DNA ligation kit (Roche Diagnostics, Mannheim) following the manufacturer's instructions and the ligation reaction was transformed into competent E.coli XL-1Blue (Stratagene, La Jolla, USA) by standard methods as described in Sambrook et al. (Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, (1989)). Selection for plasmid-bearing cells
20 was achieved by plating on kanamycin (20µg/ml) – containing LB agar (Lennox, 1955, Virology, 1:190).

The plasmid DNA preparation was carried out by methods of, and using materials from, Quiagen. Sequencing reactions were carried out as described by Sanger et al. (1977)

25 Proceedings of the National Academy of Sciences USA 74:5463-5467. The sequencing reactions were separated and evaluated by means of ABI Prism 377 (PE Applied Biosystems, Weiterstadt).

The resulting plasmid pC Phsdh metY_Mt (*Mycobacterium tuberculosis*) is listed as SEQ ID
30 NO:79. The corresponding plasmid map is shown in Figure 3.

The sequence SEQ ID NO:79 encompasses the following essential part-regions:

LOCUS pC\Phsdh\metY_Mt 6591 bp DNA circular 21-JUL-2003

	FEATURES	Location/Qualifiers
	CDS	156..1505
		/vntifkey="4"
5		/label=metY\aus\M\tuberculosis
	CDS	1855..2646
		/vntifkey="4"
		/label=Kan\R
10	CDS	4927..6048
		/vntifkey="4"
		/label=Rep\Protein
	CDS	3919..4593
		/vntifkey="4"
		/label=ORF\1
15	CDS	complement(2913..3773)
		/vntifkey="4"
		/label=Ori\EC\pMB)

20 **Example 8:** Transformation of the strain LU1479 lysC 311ile ET-16 with the plasmid pC Phsdh metY_Mt

The strain LU1479 lysC 311ile ET-16 was transformed with the plasmid pC Phsdh metY_Mt by the above-described method (Liebl, et al. (1989) FEMS Microbiology Letters 53:299-303). The transformation mixture was plated onto CM plates which additionally comprised 20mg/l kanamycin in order to achieve a selection for plasmid-containing cells. Resulting kanamycin-resistant clones were picked and isolated individually. The methionine productivity of the clones was examined in a shake-flask experiment (see Example 6). The strain LU1479 lysC 311ile ET-16 pC Phsdh metY_Mt produced significantly more methionine in comparison with LU1479 lysC 311ile ET-16.